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4-Hydroxynonenal-Induced Cell Death in Murine Alveolar Macrophages

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Oxidative stress is known to cause apoptosis in many cell types, yet the mechanism of oxidative stress-induced apoptosis is not clear. Oxidative stress has been described to cause peroxidation of polyunsaturated fatty acids. 4-Hydroxynonenal (HNE) is a diffusible product of lipid peroxidation and has been shown to be toxic to cells. In this study, the effects of HNE on isolated alveolar macrophages (AM) from two murine strains (C3H/HeJ and C57BL/6J) were examined. HNE induced the formation of protein adducts in AMs from both strains of mice in a dose-dependent manner, and the amounts of HNE-protein adducts formed in cells from both strains were very similar. In the HNE dose range from 1 to 100 μM, AMs from both strains had very little necrosis as shown by trypan blue staining. However, AMs from both C3H/HeJ and C57BL/6J mice had extensive apoptosis at 100 μM HNE, but little or no apoptosis at 25 μM HNE. Furthermore, AMs from C57BL/6J mice had significant apoptosis at 50 μM HNE while AMs from C3H/HeJ mice had no significant apoptosis at this dose. At low doses of HNE (10 to 25 μM), there was induction of heme oxygenase 1. The data indicated that HNE induces apoptosis in murine macrophages, and cells from different strains of mice have different sensitivities to the HNE-induced apoptosis. The cause of the difference in susceptibility is not known, but it is possible that different stress response and/or apoptosis-regulating proteins may be in part responsible. Our observation that a product of lipid peroxidation causes apoptosis suggested that it might be a mediator for oxidative stress-induced apoptosis. © 1996 Academic Press, Inc.

Reactive oxygen species are formed in tissues as by-products of normal oxidation reactions and can be induced by environmental agents (e.g., ozone) and toxins (e.g., paraquat). They are capable of damaging biochemical compounds such as DNA, protein, and lipid, and have been linked to many common human diseases such as cancer, heart attacks, stroke, and emphysema (Bankson *et al.*, 1993; Baruchel and Wainberg, 1992; Choi, 1992; Cross *et al.*, 1987; Favier *et al.*, 1994; Gotz *et al.*, 1994; Marx, 1987; Troy and Shelanski, 1994; Wolff, 1993; Ziv *et al.*, 1994). Oxidative stress is known to induce apoptosis in a wide variety of cultured cells (Forrest *et al.*, 1994; McConkey *et al.*, 1988; Ratan *et al.*, 1994) and is believed to cause apoptosis in various pathological conditions such as AIDS and neurodegenerative diseases (Choi, 1992; Favier *et al.*, 1994; Troy and Shelanski, 1994; Ziv *et al.*, 1994). It has been shown that cells sustain progressive lipid peroxidation following an apoptotic signal (Hockenbery *et al.*, 1993), and it has been suggested that oxidative stress is a common mediator for apoptosis (Buttke and Sandstrom, 1994). However, the essential biochemical events in oxidative stress-induced apoptosis remain to be solved. Identification of the key mediator(s) for oxidative apoptosis will contribute to the understanding of the mechanism of this process.

Oxidative free radicals are known to cause peroxidation of membrane polyunsaturated fatty acids. 4-Hydroxynonenal (HNE) is one of the major aldehydic products of lipid peroxidation and has been reported to be the most toxic aldehyde formed (Benedetti *et al.*, 1979, 1980; Benedetti and Comporti, 1987). HNE has been detected in cells under physiological conditions, and elevated HNE has been observed under oxidative conditions (Esterbauer *et al.*, 1987; Poli *et al.*, 1985; Siakotos *et al.*, 1988; Yoshino *et al.*, 1986). The general biological effects of HNE have recently been reviewed (Esterbauer *et al.*, 1991), and it has been shown to have a wide spectrum of biological effects, depending on the target cell and the concentration of HNE. In addition, it has been suggested that HNE may contribute to the cytotoxic effects of oxidative stress (Bhatnagar, 1995; Esterbauer *et al.*, 1991). The mechanism of HNE-induced cell injury is not known, but may involve adduct formation with specific cellular proteins (Esterbauer *et al.*, 1991).

A genetic model for studying the susceptibility to oxidative lung injury has been established in inbred mice. C57BL/6J mice are more sensitive to lung injury induced by ozone and hyperoxia than C3H/HeJ mice (Hudak *et al.*, 1993; Kleeberger *et al.*, 1990). The alveolar macrophage (AM) occupies a key position in mediating the interaction between environmental insults and other cell types in the lung, and is generally considered to play a central role in the regulation of the immune response to inhaled pathogens and development of inflammation and pulmonary fibrosis (Fels and

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Cohn, 1986). There is increasing evidence suggesting that the macrophage itself is a target for injury by agents known to cause pulmonary diseases (Hamilton *et al.*, 1995; Cohen *et al.*, 1991; Sarih *et al.*, 1993). Therefore, in this study, we investigated the potential role of HNE in oxidative stress-induced cell injury by examining its effects on alveolar macrophages obtained from these two strains of mice.

MATERIALS AND METHODS

Materials. 4-Hydroxynonenal was obtained from Biomol (Plymouth Meeting, PA). Medium 199 was from GIBCOBRL (Gaithersburg, MD). Fetal bovine serum (FBS) was from Sigma Chemical Co. (St. Louis, MO). Antibody against heme oxygenase 1 (pAb HO-1) was obtained from Stress-Gen (Victoria, BC, Canada). Antibody against HNE-protein adducts was kindly provided by Dr. Luke Szweda (Case Western Reserve University) (Uchida *et al.*, 1993). Antibody against actin (mAb actin), horseradish peroxidase-linked anti-rabbit Ig whole antibody (from donkey), and horseradish peroxidase-linked anti-mouse Ig whole antibody (from sheep) were obtained from Amersham (Arlington Heights, IL).

Isolation of mouse alveolar macrophages. C57BL/6J and C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). After arrival, mice were housed for at least 3 days to acclimate. Mice were anesthetized with 60 mg/kg sodium pentobarbital and exsanguinated by severing dorsal arteries. Then, the lungs were removed and lavaged five times with a single 1.0-ml aliquot of phosphate buffer solution (pH 7.2), and the lavage fluid was collected on ice. Cells were recovered by centrifuging the lavage fluid at 1500 rpm for 7 min. Cells were resuspended in Medium 199, and cell counts were determined using a Coulter counter. Cell viability was determined by trypan blue exclusion. Typical cell recoveries for murine lavage were approximately 0.5×10^6 cells per mouse, 95% alveolar macrophages and 85–95% viability. For each experiment, cells were pooled from 12 mice to decrease experimental variability and provide enough material to conduct the studies described below.

Cell treatment. Freshly isolated lung cells suspended in Medium 199 were incubated with different concentrations of HNE for 10 min at room temperature. After the incubation, heat-inactivated fetal bovine serum (FBS) was added to the medium to a final concentration of 10%. Cells were cultured in suspension by slow end-to-end tumbling (Labquake Shakers, Labindustries) in sterile polypropylene tubes (PGC Scientific) with or without HNE at 37°C for 5 hr before harvesting. Following treatment, cells were examined by Western analysis (for HNE-adducts and HO-1 expression) and for cell injury (necrosis and apoptosis).

Protein sample preparation. Cells were washed once with phosphate-buffered saline (PBS) (pH 7.2) and centrifuged at 1500 rpm for 7 min at 4°C. The cells were then solubilized in Laemmli sample buffer (2% SDS, 10% glycerol, 60 mM Tris–HCl, pH 6.8, 0.1 M DTT, 0.01% bromphenol blue), sonicated for 30 sec with an ultrasonifier cell disrupter (Heat System-Ultrasonics, Inc., Farmingdale, NY), and heated in boiling water for 5 min prior to storage at −20°C.

SDS–PAGE and Western blotting. Protein samples were analyzed with SDS–gel electrophoresis (12% Ready Gels, BioRad, Hercules, CA). Resolved proteins were transferred to nitrocellulose membranes (Amersham) using Trans-Blot Electrophoretic Transfer Cell (BioRad). The membranes were incubated for 16 hr at 4°C in blocking buffer (5% Blotto in TNA buffer containing 10 mM Tris–HCl, pH 7.2, 150 mM NaCl) and were then incubated with primary antibody (1:2000 for HO-1 pAb, 1:500 for HNE pAb, and 1:2000 for actin mAb) for 16 hr at 4°C. The membranes were washed extensively with TBST (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween, pH 8.0), incubated with second antibody conjugated to horseradish peroxidase (Amersham) at 1:10,000 dilution for 1 hr at room temperature, and washed extensively with TBST. Enhanced chemiluminescence detection (Amersham) followed by autoradiography (Hyperfilm-ECL, Amersham) was used to visualize the proteins.

Trypan blue exclusion assay. Cells in suspension were treated with an equal volume of trypan blue solution (0.5% trypan blue in 0.85% saline, Flow Laboratories, McLean, VA) for 2 min and examined under light microscopy. Only necrotic cells internalize the dye and become stained. At least 200 cells were counted for each group.

Apoptosis assays. Macrophage apoptosis induced by HNE was examined by a combination of Wright's Giemsa staining (Curtin Matheson Scientific, Inc., Houston, TX) and the detection of DNA fragmentation in the cells.

For Wright's Giemsa staining, cells were suspended in PBS (pH 7.2) at room temperature for 5 min, cytocentrifuged onto charged microscope slides (Fisher Scientific) at 1500 rpm for 5 min, fixed in cold methyl alcohol for 5 min, stained in Wright's Giemsa stain for 2 min, and then stained in buffered Wright's Giemsa stain (pH 6.8) for 4 min. The slides were dried in air and examined by light microscope.

For the detection of nucleosomes in cytoplasmic fractions of the cells, the samples were processed and analyzed using the Cell Death Detection ELISA kit (Boehringer Mannheim) according to the manufacturer's protocol. The assay is based on the quantitative sandwich-enzyme-immunoassay principle using monoclonal antibodies directed against DNA and histone. From each sample, 1×10^5 cells were processed, 5000 cells were used for each reaction, and triplicate reactions were performed for each sample.

To visualize DNA fragmentation, genomic DNA was isolated from treated cells using the DNA ISOLATOR (Genosys, Woodland, TX) and 3′-end labeled with [α-^{32}P]dCTP (ICN) by incubation of 1 μg of DNA in 50 μl of reaction buffer (50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 200 μM dATP, 200 μM dGTP, 200 μM dTTP, 2 μl of [α-^{32}P]dCTP, 2 U klenow) at 37°C for 30 min. The same amount of [α-^{32}P]dCTP-labeled DNA (50 ng) for each sample was loaded onto a 2% agarose gel and run at 5 V/cm for 5 hr in 40 mM Tris–acetate buffer, pH 8.0, with 1 mM EDTA. The gel was dried at 60°C under vacuum in a gel drier and then exposed to X-ray film.

Large DNA fragmentation was assayed by pulsed-field electrophoresis as described by Huang and Plunkett (1995). In brief, cells were harvested and washed with PBS before fixation in 0.6% low melting agarose gel in gel plug molds (BioRad). The cells were lysed in the gel plugs by incubation with lysis buffer (1% sarkosyl, 50 mM EDTA, 50 mM Tris–HCl, pH 7.8) at 45°C for 16 hr. The gel plugs were washed with TE buffer and run onto 1% agarose gel using CHEF-DR III system (BioRad). The DNA was visualized by ethidium bromide staining of the gel.

Statistical analysis. Statistical differences between exposure groups were determined by a one-way analysis of variance (ANOVA) followed by Student Neuman–Keuls tests. Values are reported as the means ± SEM. Differences were considered statistically significant at $p \leq 0.05$. Data in Figs. 2 and 4 represent an $n = 2$ (each n represents cells pooled from 12 mice) and consequently are presented without statistics.

RESULTS

Formation of HNE-Modified Proteins in HNE-Treated Cells

It has been shown that HNE forms protein adducts in cells and the formation of specific HNE-protein adducts may account for its cytotoxicity (Esterbauer *et al.*, 1991). To determine whether differences exist in the response of lung cells from both strains of mice to HNE, macrophages from

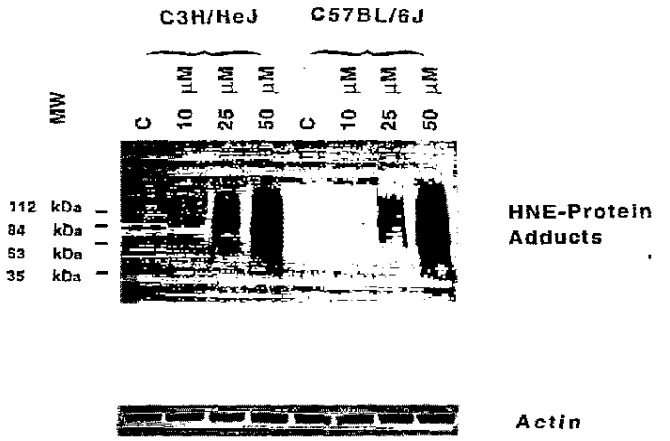

FIG. 1. Formation of HNE-protein adducts in treated cells. Cells were cultured in suspension with or without HNE at 37°C for 5 hr before harvesting and were examined for HNE-protein adducts by Western analysis. C represents cells from C3H/HeJ or C57BL/6J mice that were cultured in medium without HNE. 10 μM, 25 μM, and 50 μM represent the concentrations of HNE.

both strains were treated with different doses of HNE for 5 hr and then analyzed for HNE-protein adducts. Figure 1 indicates that macrophages from both strains form qualitatively similar amounts of HNE-protein adducts, and that the pattern of HNE-protein adducts is similar. This result suggested that cells from both strains of mice have similar levels of antioxidant defense mechanisms (e.g., nonprotein thiols such glutathione and cysteine, and glutathione *S*-transferase, etc.). Consistent with this speculation, no difference of GSH levels between the two murine strains was detected (data not shown).

Necrosis in HNE-Treated Cells

The biological effects of HNE on macrophages from both strains of mice were consequently examined. Cell viability was assayed after the HNE treatment. Using trypan blue exclusion as an assay of cell necrosis, no change in cell viability was observed in either strain of mice at doses up to 100 μM HNE (Fig. 2). At HNE concentrations of 200 μM and higher, there was similar marked necrosis in the HNE-treated cells from both murine strains (Fig. 2).

Apoptosis in HNE-Treated Cells

Apoptosis was examined by light microscopy of cells treated with HNE at doses ($\leq$100 μM) where no obvious necrosis was detected (Figs. 3 and 4). HNE caused no apparent apoptosis at concentrations of 25 μM or lower in either strain, but induced the formation of a large number of apoptotic cells at 100 μM in both strains of mice (78.2 and 69.4% for cells from C57BL/6J and C3H/HeJ, respectively). At 50 μM HNE, cells from C3H/HeJ mice showed no significant signs of apoptosis, while cells from C57BL/6J mice had significant apoptosis (15% in treated cells vs 1% in control cells). HNE induced distinctive morphological changes characteristic of apoptotic cells, including cell shrinkage, nuclear chromatin condensation, and segmentation of the nucleus (Fig. 3). Consequently, even though HNE caused similar protein adducts and necrosis in cells from both strains of mice, cells from C57BL/6J mice were more sensitive to apoptotic injury by HNE.

To confirm that the cells were undergoing apoptosis following HNE treatment, DNA fragmentation was examined using different approaches (Fig. 5). Cytosolic histone-bound DNA fragments were detected by Cell Death Detection ELISA (Fig. 5A). A significant increase in DNA fragmentation was observed in cells in a dose-dependent manner. Furthermore, the increase in DNA fragmentation correlated with the degree of apoptosis in both murine strains. These data confirmed the morphological analysis and reaffirmed the difference in strain sensitivity.

Apoptosis is usually accompanied by internucleasomal DNA fragmentation resulting in a DNA ladder of multiples of 180–200 base pairs. However, classical internucleasomal DNA fragmentation (DNA ladder) was not present in these apoptotic cells treated with 100 μM HNE (Fig. 5B). Accordingly, DNA fragmentation was examined by pulsed-field gel

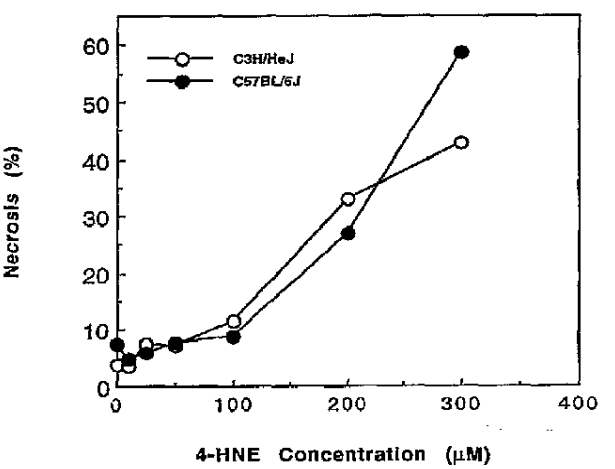

FIG. 2. Necrosis in HNE-treated cells. The cells were exposed to trypan blue dye (0.04% in PBS), placed on a hemocytometer, and examined under light microscopy. Only necrotic cells internalize the dye and become stained. Two hundred random cells were counted after each treatment and the percentage of stained cells is expressed as the percentage of necrosis for any given condition. The percentage of necrosis for each treatment was the average of two independent experiments. For each experiment, 12 mice from each strain were lavaged and the cells were pooled for each mouse strain.

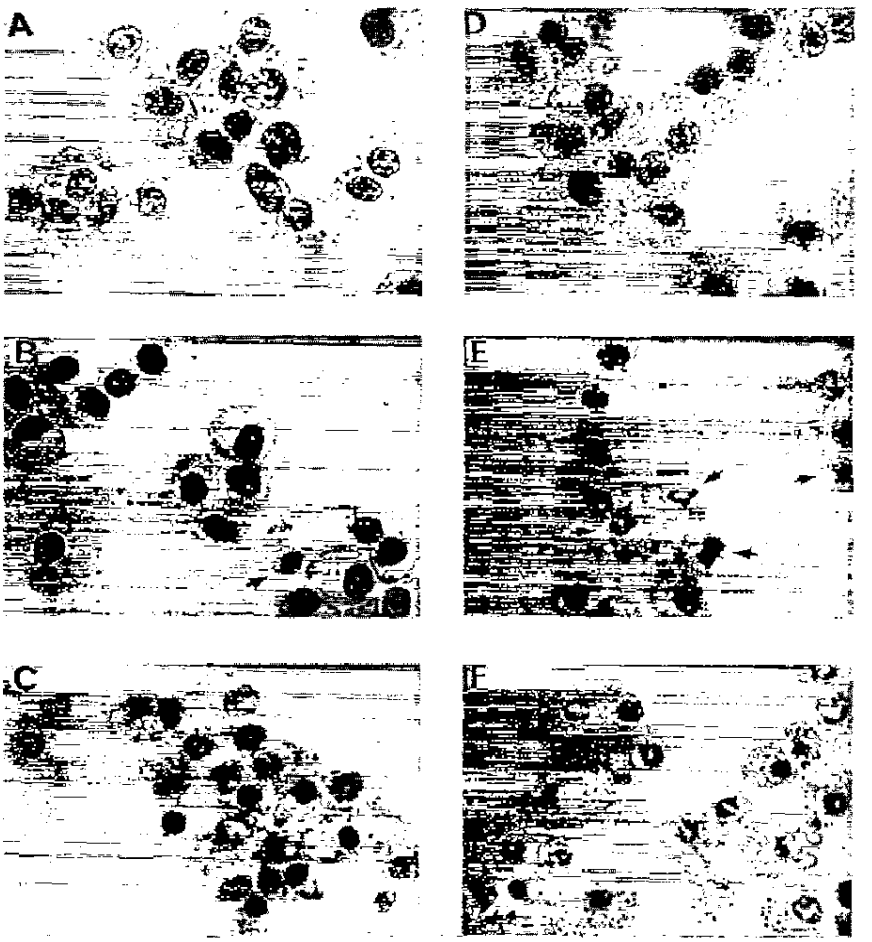

FIG. 3. Apoptosis in HNE-treated cells. Macrophage apoptosis induced by HNE was examined by Wright's Giemsa staining. Cells were treated with increasing concentrations of HNE as described in the legend to Fig. 1 and harvested after 5 hr. A, B, and C are cells from C3H/HeJ mice; D, E, and F are cells from C57BL/6J mice. A and D are the control cells, B and E are cells treated with 50 μM HNE, and C and F are the cells treated with 100 μM HNE. Arrows point to the apoptotic cells in B and E.

electrophoresis in order to determine whether HNE apoptosis is associated with the formation of large DNA fragments. As shown in Fig. 5C, high-molecular-weight DNA fragments (50 kb) were detected in these apoptotic cells and appeared to be more pronounced in cells from C57BL/6J mice. Taken together, the data demonstrated that HNE-induced cellular apoptosis was associated with large DNA fragmentation in both strains of mice, with cells from C57BL/6J mice being more sensitive.

Stress Response in HNE-Treated Cells

It is known that oxidative stress causes the induction of stress proteins (Cajone and Bernelli-Zazzera, 1988; Jornot *et al.*, 1991; Keyse and Tyrrell, 1987; Yamaguchi *et al.*, 1993). HNE (100 to 200 μM) has been reported to induce the synthesis of a 31-kDa protein and a 70-kDa (HSP70) protein in rat hepatocytes and rat hepatoma cells MH_1C_1 (Cajone and Bernelli-Zazzera, 1988). Since oxidative stress has been shown to induce HO-1 (Applegate *et al.*, 1991; Kutty *et al.*, 1988; Stocker, 1990; Stocker *et al.*, 1987), it raised the possibility that the 31-kDa protein could be HO-1. Therefore, we examined whether HNE could induce HO-1 in both murine strains. As shown in Fig. 6, HO-1 was induced in cells treated with HNE at doses below those causing cell injury, whereas HO-1 was not detectable at higher doses of HNE when the majority of cells were apoptotic. Although the role of HO-1 in HNE-induced apoptosis is not clear, it was evident that its synthesis occurred at low concentrations of HNE (5 and 10 μM; data not shown), again consistent with the spectrum of effects of oxidative stress

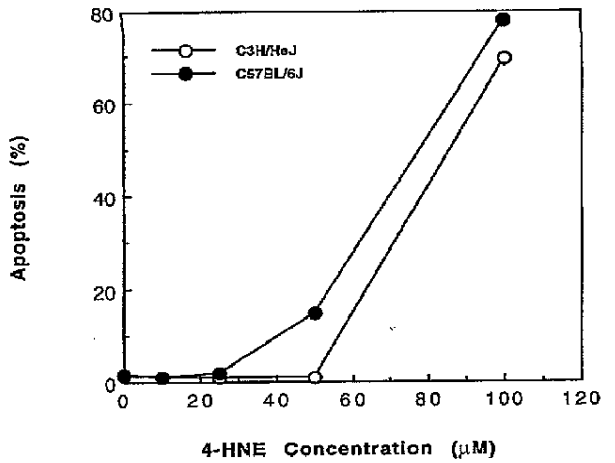

FIG. 4. Percentage of apoptotic cells following HNE treatment. At least 800 cells were counted for each treatment as described in the legend to Fig. 3. The percentage of apoptotic cells is expressed as a percentage of apoptosis. The percentage of apoptosis for each treatment was the average of two independent experiments. For each experiment, 12 mice from each strain were lavaged and the cells were pooled for each mouse strain.

on cells. It was also evident from Fig. 6 that there was no induction of HO-1 in the cells from C57BL/6J mice at 50 μM HNE, whereas, in the C3H/HeJ cells, the HO-1 level was still elevated, consistent with the increased sensitivity of cells from C57BL/6J mice to oxidative injury.

DISCUSSION

Oxidative free radical-induced lipid peroxidation causes the formation of reactive aldehydes. These aldehydes have longer biological half-lives than free radicals and can diffuse from their site of formation to reach distant targets and cause cellular damage. It has been suggested that these aldehydes may act as second toxic messengers of lipid peroxidation (Esterbauer *et al.*, 1991).

In the current studies, HNE induced a stress response, apoptosis, or necrosis, depending on the concentration. This pattern of response is characteristic of oxidative stress (Dypbukt *et al.*, 1994; Lennon *et al.*, 1991). Since HNE has been shown to be formed during oxidative stress and mimics the effects of oxidative stress, it may be a mediator for oxidative stress-induced cell damage. The fact that HNE causes apoptosis in treated cells provides a linkage between oxidative stress and apoptosis and suggests that HNE might be a mediator for oxidative stress-induced apoptosis.

The concentrations of HNE required to induce significant apoptosis in the macrophages are in the range of 50 to 100 μM. It is unlikely that HNE can reach an overall concentration of 100 μM in cells or organs. However, it is possible that high concentrations of HNE can build up locally near or within peroxidizing membranes. It has been suggested that the HNE concentration in the lipid bilayer of isolated peroxidizing microsomes can reach 4.5 mM (Koster *et al.*, 1986), and it has been proposed that HNE may cause cell injury by attacking target proteins within the membrane. Furthermore, lipid peroxidation is a complex process, and many other reactive aldehydes are formed. Therefore, the combination of all these aldehydes (HNE and others) is most likely to cause apoptosis under pathological conditions.

The detection of cytoplasmic histone-associated DNA fragments by the Cell Death Detection ELISA allowed a quantitative measurement of the degree of DNA fragmentation in the apoptotic cells. Our results showed a good correlation between the degree of apoptosis by morphological analysis and the degree of DNA fragmentation measured by the ELISA procedure. Also, the Cell Death Detection ELISA seems selective for measuring apoptotic DNA fragmentation in our experiments, since very low readings were observed at high HNE concentrations when cell necrosis was dominant (data not shown). Conversely, the trypan blue exclusion assay appears to be selective for necrosis since no detectable positive staining was observed at HNE doses when massive apoptosis was evident.

In our experiments with HNE, we observed high-molecular-weight (50 kb) DNA fragments, but not internucleosomal DNA fragments (DNA ladders) in the apoptotic cells. This result is consistent with the rational that high-molecular-weight DNA fragmentation is essential for apoptosis and precedes the formation of internucleosomal DNA fragmentation (Weaver *et al.*, 1993; Walker *et al.*, 1994). It is not clear how HNE prevented the internucleosomal fragmentation of DNA. A possible explanation for this phenomenon is that since HNE is thiol reactive (Esterbauer *et al.*, 1991), it may inactivate cysteine proteases involved in internucleosomal DNA cleavage (Weaver *et al.*, 1993). Also, the lack of DNA ladder formation could be due to the short culturing time in our studies. We detected internucleosomal DNA fragmentation in human alveolar macrophages treated with HNE for 24 hr, but not in those treated for 6 hr (data not shown). However, the short survival time of murine alveolar macrophages in culture makes it impractical to assay for HNE-induced DNA fragmentation for much longer than 5 hr.

It is not known how HNE induces apoptosis. HNE reacts with protein thiols and can form adducts with cysteine, lysine, and histidine as well as other residues in target proteins (Esterbauer *et al.*, 1991; Uchida and Stadtman, 1993). The formation of HNE-adducts in key proteins could lead to the activation of apoptosis. Also, HNE disturbs calcium homeostasis (Benedetti *et al.*, 1984; Griffin and Segall, 1987) and damages microtubules (Gabriel *et al.*, 1985; Olivero *et al.*, 1992; VanWinkle *et al.*, 1994), both of which could lead to

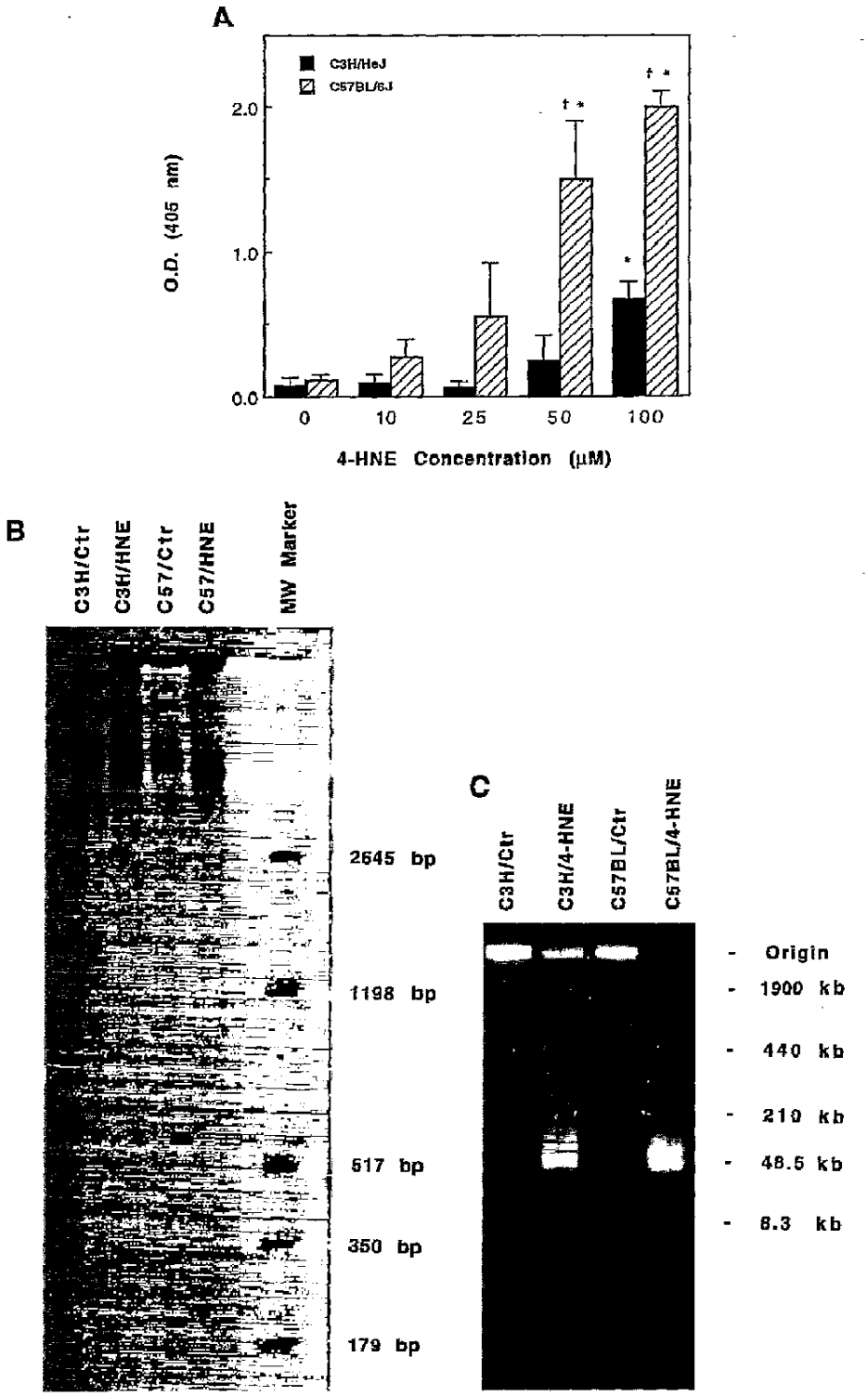

FIG. 5. DNA fragmentation in HNE-treated cells. (A) Cytosolic histone-bound DNA fragments detected by Cell Death Detection ELISA. 1×10^5 cells from each condition were processed, 5000 cells were used for each reaction, and triplicate reactions were performed for each condition. The results are from three separate experiments ($n = 3$). *Statistically significant difference between treatment and control group ($p < 0.05$); †statistically significant difference in cells between the two murine strains under the same condition ($p < 0.05$). (B) Electrophoretic analysis of genomic DNA. Cells from both murine strains were treated with 100 μM HNE and analyzed for DNA ladder formation. C3H/Ctr, C3H/HNE, C57/Ctr, and C57/HNE represent DNA from control C3H/HeJ cells, 100 μM HNE-treated C3H/HeJ cells, control C57BL/6J cells, and 100 μM HNE-treated C57BL/6J cells, respectively. (C) Pulsed-field gel electrophoresis of DNA from HNE-treated cells. C3H/Ctr, C3H/HNE, C57/Ctr, and C57/HNE represent DNA from control C3H/HeJ cells, 100 μM HNE-treated C3H/HeJ cells, control C57BL/6J cells, and 100 μM HNE-treated C57BL/6J cells, respectively.

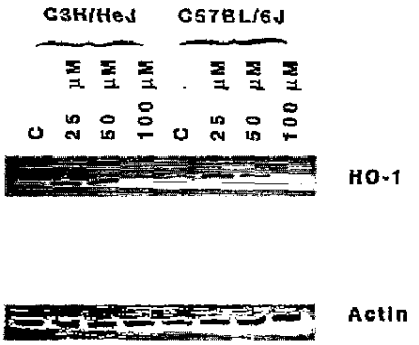

FIG. 6. Heme oxygenase 1 (HO-1) expression in HNE-treated cells. Lung cells from both murine strains treated with increasing concentrations of HNE were harvested, and their cellular proteins were processed and examined for heme oxygenase (HO-1) and control actin level by Western analysis. C represents cells from C3H/HeJ or C57BL/6J mice cultured in medium without HNE. 25 μM, 50 μM, and 100 μM represent the concentrations of HNE. The figure is representative of three independent experiments. For each experiment, 12 mice from each strain were lavaged and the cells were pooled for each mouse strain.

subsequent apoptosis (Duvall and Wyllie, 1986; Lam *et al.*, 1993; Martin and Cotter, 1990a,b; Nicotera and Rossi, 1994; Takano *et al.*, 1993). Furthermore, HNE has been reported to cause DNA strand breaks (Brambilla *et al.*, 1986; Eckl and Esterbauer, 1989; Esterbauer *et al.*, 1990), which could lead to the activation of poly(ADP-ribose) transferase and the accumulation of p53 protein; both proteins are associated with apoptosis (Clarke *et al.*, 1993; Jones *et al.*, 1989; Lowe *et al.*, 1993; Rice *et al.*, 1992). Multiple factors may be involved in HNE-induced apoptosis, and the difference in the murine strain sensitivity to oxidative injury may be related to differences in apoptosis-regulating proteins (bcl-2, p53, Fas, Bax, BAD, etc.) in cells between the two murine strains.

Our results demonstrated that HNE can induce HO-1 expression consistent with oxidative stress. Furthermore, this occurs at HNE doses below those causing apoptosis. In addition, the pattern of HO-1 expression was different between the cells from two murine strains. The difference in stress response (HO-1 induction) may in part account for the difference in sensitivities to oxidative injury between the two strains of mice, because HO-1 is proposed to be an important cellular antioxidative protein (Applegate *et al.*, 1991; Kutty *et al.*, 1988; Stocker, 1990; Stocker *et al.*, 1987).

In vivo studies using different inbred murine strains indicated that the strain difference in susceptibility to ozone- and hyperoxia-induced lung injury is under genetic control (Hudak *et al.*, 1993; Kleeberger *et al.*, 1990, 1993). Our *in vitro* results with HNE suggest that genetic differences in strain sensitivity occur at the cellular level.

In summary, our data indicate that 4-hydroxynonenal is capable of inducing a stress response, apoptosis, or necrosis at increasing concentrations. The observation that a product(s) of lipid peroxidation induced apoptosis provides a possible mechanism to account for oxidative stress-induced apoptosis.

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